THE HYDROLYSIS OF HIGHER FATS IN EGG-SECRETION.

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I.

In four recent publications ('15, '18, '21, and '22) Richards, Miss Woodward, and I have dealt with the enzymic properties of *Arbacia* egg-secretion. It was shown, first of all, that radiation affects these secretions as though enzymes were present; next, there was precipitated a body, lipolysin, which, like unmodified exudate, was subsequently shown to accelerate the hydrolysis of ethyl butyrate. Finally, in my last paper ('22), I reported the synthesis of butyric ester.

On the basis of these results, we may assume that lipolysis plays a rôle in the initiation of development. The position is greatly strengthened by the fact that normal eggs may be completely sterilized if their secretions are removed by short exposure to charcoal ('21). Nevertheless the view that initiatory changes are somehow linked with the activities of a lipolytic enzyme requires further evidence. So far the results with ethyl butyrate are the clearest of all. However, if these were due, as they might be, to a relatively specific esterase, the fact, though interesting from other points of view, would be difficult to fit into a theory of fertilization. In order that lipolysis may find a place as part of the mechanism of initiation, it must first be shown that the enzymes present in egg-secretion actually affect the hydrolysis of higher fats.

II.

Preliminary tests were made with olive oil. This was carefully neutralized with dilute NaOH and subsequently extracted with fresh water, and later with ether ('09). The oil was then ready for use.

If egg-secretion influences the hydrolysis of olive oil, the effect, under the proper conditions, might be rendered apparent through

changes localized at the oil-exudate interface. Here important disturbances should take place, although mere volumetric changes are not likely to be reliable. The diffusion of the glycerine set free in hydrolysis would, of course, result in a decrease in the volume of the oil drops. On the other hand, the production of oleic acid with its great affinity for oxygen and its capacity for breaking down into lower fatty acids might easily balance, or even overbalance, the loss resulting from the solubility of the glycerine in egg-water. Conceivably, then, the drops might decrease in volume, increase in volume, or even remain constant.

Nevertheless, if hydrolysis takes place, a lowering of the surface tension at the oil-exudate interface is to be expected, and, regardless of volumetric considerations, should bring about a concentration at the phase-boundary of substances either in solution or in suspension within the oil. If such substances happen to be insoluble in egg-secretion, one may likewise expect their precipitation immediately about the periphery of a drop from which glycerine and possibly the oxidation products of oleic acid, are diffusing outward.

I used two indicators; in some tests Sudan III. was dissolved in the olive oil, in others pulverized charcoal was suspended. A sharp phase-boundary, permitting a clear focus under the microscope, was secured by placing small discs of the oil on the surface of the exudate. The observations were checked by comparisons made on sea-water and distilled, as well as by the use of oil without indicators. Additional controls were carried on with solutions of pancreatin and the commercial Holadin.

With discs of plain olive oil on sea-water, there is some marginal effect. This is indicated by the rather rapid development of a moderately irregular outline. On distilled water the discs maintain their smooth circular form almost perfectly during the first few hours of an experiment. The differences in these two cases are no doubt traceable to the sea salts.

If egg-secretion is used in place of sea-water, the oil discs in the course of an hour take on a form suggestive of a circular saw whose projecting teeth are in a state of active disintegration. Small particles can be seen breaking off in large numbers until the originally sharp outline of the disc becomes quite obscured. Simi-

lar effects were noticed on sea-water, but they develop less rapidly and, within the time limit of these experiments, never equaled the effects gotten with egg-secretion.

With Sudan III., in the early stages of an experiment, peripheral elimination and precipitation of the dye on either sea-water or distilled is questionable. On egg-secretion, however, there is no doubt. The outlines of the discs become exceedingly irregular; there is marked surface concentration of the stain, and very minute particles of it are precipitated densely about the periphery. After 12 hours the oil discs are quite gone. In place of them one finds irregular islands, stained with Sudan III. After 24 hours a rancid odor is noticeable.

With pulverized charcoal exactly comparable results can be gotten, only the surface changes are even more striking. After 24 hours the islands on egg-water can be distinguished macroscopically from their controls, partly because of their greater irregularity, and partly because they have become vesiculated.

If the differences noted are due to the activities of a lipolytic ferment, then solutions known to contain fat-splitting enzymes should reproduce these differences. Attempts were made with two products, the one labeled Pancreatin and of unknown origin, the other a Parke-Davis preparation with the trade name Holadin. Both of these, especially in experiments in which charcoal was used, gave very striking effects and, after 24 hours, the irregular vesiculated islands could be distinguished even macroscopically from their controls.

III.

In these first experiments I made no attempt to control bacterial action. Whatever may be true after six, twelve, or twenty-four hours, the earliest noticeable differences between the oil discs on exudate and on sea-water became apparent so quickly that bacterial digestion seems unlikely. This fact, therefore, warrants a more careful examination of the fat-splitting properties of the secretion.

I now prepared exudate as free as possible from bacterial contamination. The females were thoroughly washed in running fresh water and carefully dried. Only shed eggs were used and these in filtered sea-water. The secretion itself was filtered several

times through Chardin paper and the remaining infection controlled by the addition of chloroform. KCN was not employed because it checks the action of certain lipases.

With exudates of this type I shook up varying proportions of either olive oil or whale oil and at stated intervals attempted to titrate the systems with NaOH N/40 back to the specific turning points of such indicators as phenolphthalein, neutral red, or litmus. On account of the buffer effects and saponifications due to the salts present in both secretion and sea-water, such titrations, even if otherwise reliable, can not disclose the total acidity.

But there are other difficulties. Digests of the type here under consideration are almost proverbially mean. In addition, these particular systems in egg-secretion, in the course of an hour or two, underwent serious physical changes. At the beginning of an experiment, oil and secretion, after shaking, would separate promptly, but after standing for the stated times at 20° C. the separation was much less complete. After four hours even moderate shaking imparted to the systems a stability almost jelly-like.

Under these circumstances, even with the addition of neutral alcohol ('03), titration gave highly variable results, and hence nothing in the nature of a curve showing the course of the reaction can be plotted. However, if we take the most reliable titrations—*i.e.*, those made upon volumes measured before the physical changes previously referred to had taken place—certain very definite comparisons are possible.

Series *A*.....40 c.c. secretion	40 c.c. sea-water
10 c.c. olive oil	10 c.c. olive oil
After 45 hrs.....10 c.c.=2.7 c.c. NaOH *N*/40	10 c.c.=1.9 c.c. NaOH *N*/40
Series *B*.....150 c.c. secretion	150 c.c. sea-water
25 c.c. olive oil	25 c.c. olive oil
After 30 min.....10 c.c.=1.5 c.c. NaOH *N*/40	10 c.c.= .4 c.c. NaOH *N*/40
Series *C*.....90 c.c. secretion	90 c.c. sea-water
25 c.c. olive oil	25 c.c. olive oil
After 30 min.....10 c.c.=1.8 c.c. NaOH *N*/40	10 c.c.=1.3 c.c. NaOH *N*/40

These values indicate the number of c.c. of NaOH N/40 necessary to return the several systems to P_{H_8}, using phenolphthalein as the indicator. The relatively high acidity found in the controls is a disturbing factor, but unavoidable, since oleic acid is never en-

tirely absent in a "neutral" oil; is, moreover, constantly being produced; and, as constantly oxidized to dioxystearic and, very likely, other lower fatty acids. The differences between the digests and the controls, however, remain significant.

IV.

By far the most fruitful observation was made, not on the digests themselves, but on the litmus which in certain cases was used as the indicator. As compared with the controls in sea-water and oil, and in exudate without oil, neutral litmus, when shaken up in mixtures of secretion and either whale oil or olive, instantly becomes pinker. After two hours the pink is distinctly intensified, and after twelve, such digests stand out sharply from their controls.

Within twenty-four hours all color, including the pink, disappears in the oil-secretion digests. If more litmus is now added, it again turns pink, and, in the course of time, fades out completely. The controls, on the other hand, even after forty-eight hours, still appear "neutral," with perhaps only the faintest leaning toward pink.

It is plain that the litmus is not functioning here as a direct indicator of acidity. Very likely the change to pink and the final complete decolorization are the first and last visible steps in a process of reduction. But why, we may ask, the difference between the secretion digests with neutral oil and the controls? If the explanation is to be found in the superior reducing powers of the oil-secretion systems, then these must be producing a substance capable of binding oxygen. Moreover, they must be producing this material at a rate far in excess of the rate in the controls.

Now, if the oils in the presence of secretion are undergoing ordinary lipolytic cleavage, one of the reaction products must be oleic acid, and this, as is well known, absorbs oxygen with great ease. Is it possible, then, to attribute the decolorization of the litmus to the reducing powers of oleic acid? We can only do this if we can first show that oleic acid is present in higher concentration in the digests than in the controls; and, if we succeed in proving this, we shall incidentally also furnish the proof that the only source of oleic acid—the neutral oil—is undergoing accelerated hydrolysis in the presence of egg-secretion.

V.

With this idea in mind, I prepared a special set of tubes, using two volumes of egg-secretion, one volume of either neutral olive or whale oil, and one volume of a neutral litmus solution. The tubes were "chloroformed" as before, then stoppered and shaken.

The secretion used in these tests was made with every precaution possible, and I kept a microscopic check on each tube. In a few instances I could find no organisms whatever either at the beginning or at the end of the period of digestion; in other cases the number was too slight to account for the results, since control tubes, deliberately infected to the point of cloudiness, required forty-eight hours to produce a barely noticeable reduction of the litmus, whereas in the oil-secretion digests the reduction always began instantaneously.

Tubes without chloroform became turbid within forty-eight hours and developed the unmistakable rancid odor. In the tubes with bacterial growth inhibited no rancid odor could be noticed on account of the masking effect of the chloroform. This necessitated some other indicator for the presence of oleic acid.

The test finally chosen was based on a statement of Hammarsten's ('12), and depends on the fact that oleic acid in the presence of cane sugar and sulfuric acid develops a purple color. The usual method was to place a drop of the digest on a slide, to mix with this a drop of saturated solution of cane sugar, and to add from 2 to 5 drops of concentrated sulfuric acid.

Care must be taken not to add the sulfuric too quickly, or to use too much, for if the sugar breaks down suddenly, the carbon set free obscures the reaction in the oleic acid. With these precautions, however, the test is delicate and very reliable. The change in the oleic-acid globules can easily be followed under the microscope. It begins as a slight discoloration, succeeded by pink—rose—and finally a deep purple. At 20° C. the reaction is slow with freshly formed oleic acid, but proceeds more rapidly after the oleic has been exposed for some time to the air.

By this method it was possible to demonstrate the presence of oleic acid in the digests in which chloroform had masked the rancid odor. Moreover, the concentration of oleic acid, as shown by the number, size, distribution, and depth of color of the purple glob-

ules, left no doubt that hydrolysis was proceeding at a faster rate in secretion than in sea-water. In the controls an occasional globule did turn purple, but this was to be expected from our inability to prepare, or, for that matter, even to keep, a fatty oil absolutely neutral.

VI.

It may be taken as demonstrated, then, that *Arbacia* egg-secretion has the power to hydrolyze higher fats. Since neither whale oil, olive oil, nor ethyl butyrate occur in sea-urchin eggs, we must conclude that the lipase present is non-specific.

The question now remains whether the lipolysin first isolated by Miss Woodward also affects the hydrolysis of higher fats.

To determine this, I repeated the experiments described in the preceding section, using, in place of egg-secretion, sea-water solutions of the lipolysin precipitate. The concentrations employed were, roughly, 25 milligrams in 10 c.c. of solvent. To report the results in detail would be a mere reiteration of the experiments with egg-secretion. In every case the evidence for hydrolysis was positive, and leads to the conclusion that the precipitated lipolysin is, or contains, that enzyme which in unmodified egg-water is responsible for the hydrolysis of the higher fats.

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